Postnatal Development of Lymphatic Vessels and Their Smooth Muscle Cells in the Rat Diaphragm: A Confocal Microscopic Study

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Summary. This paper reports on how lymphatic vessels and their smooth muscle cells develop in the diaphragm of postnatal rats. Lymphatic endothelial cells in the diaphragm were labeled by an intraperitoneal injection of Dil-labeled acetylated low-density lipoprotein (Dil-ac-LDL). During postnatal week 1, Dil-ac-LDL was detected in many free cells in addition to distinct endothelial cells that formed lymphatic vessels. Occasionally, saccular lymphatics isolated from previously formed lymphatics were recognized; these were referred to as lymphatic islands. The Dil-ac-LDL-labeled free and lymphatic endothelial cells showed immunoreactivity for CD34 and Flt-4, but most of them did not express either OX 62 or ED-1 immunoreactivity, with only some showing ED-1 immunoreactivity. This suggests that most of the Dil-ac-LDL-labeled elements were lymphatic endothelial cells, and that some were macrophages. After postnatal week 1, the Dil-ac-LDL-positive cells were restricted to lymphatic vessels. Until postnatal week 6, lymphatic vessels increased as the diaphragm enlarged. Towards the end of postnatal week 2, free cells expressing α-smooth muscle actin (α-SMA) immunoreactivity increased in the diaphragm, and some of these were in contact with lymphatics. A coarse plexus of smooth muscle cells surrounding the lymphatic vessels first appeared at postnatal week 2, and this plexus became denser with age. Our findings indicate that lymphatic vessels are formed not only by sprouting from previously formed lymphatic vessels but also by migrating endothelial cells, and that smooth muscle cells may be differentiated from mesenchymal cells to form a plexus surrounding the lymphatic vessels.

An understanding of the mechanisms of lymphangiogenesis holds importance for future lymphedema therapy and the prevention of cancer metastasis. In 1900, Sabin (1900) suggested that, early in development, isolated primitive lymph sacs originate from endothelial budding from veins. The peripheral lymphatic system spreads from these lymphatic sacs by endothelial sprouting into the surrounding tissue and organs (Sabin, 1900, 1902). This model of lymphatic development appears to be supported by recent studies. Some authors have reported that vascular growth factor receptor-3 (VEGFR-3; also known as Flt-4) is initially expressed in angioblasts of head mesenchyme, dorsal aorta, cardinal vein, and alantois in mice, then in the developing venous and presumptive lymphatic endothelium at embryonic day 12.5, and finally becomes restricted to the lymphatic endothelium in adult tissue (Kaipainen et al., 1995; Dumont et al., 1998; Valtolina et al., 1999). On the other hand, Huntington and McClure (1907) suggested that the initial lymph sacs arising in the mesenchyme are independent of veins and secondarily establish venous connections. Our previous study by enzyme-histochemistry for 5'-nucleotidase (5'-Nase) (Wachstein and Meisel, 1957) and by electron microscopy have shown that lymphatics in the rat diaphragm develop primarily by sprouting from previously formed lymphatics (Shao et al., 1998). However, during those experiments, we noticed that there were many free cells around the lymphatics densely expressing 5'-Nase, which suggested these to be additional candidate cells for lymphangiogenesis. Enzyme-histochemistry for 5'-Nase is widely used to demonstrate lymphatic endothelial cells because they contain 5'-Nase more than other kinds of cells, especially blood vascular endothelial cells (Werner et al., 1987; Kato and Miyauchi, 1989). In the present study, we have examined whether or not free endothelial cells or their progenitors participate in the formation of lymphatics in the developing diaphragm. In addition, we have also studied the process of smooth muscle cell aggregation around lymphatic vessels, because it is unknown when and how smooth
muscle cells appear around the lymphatic vessels. Lymphatic endothelial cells in the diaphragm of neonatal and adult rats were labeled by an intraperitoneal injection of Dil-labeled acetylated low density lipoprotein (Dil-ac-LDL); diaphragms were immunohistochemically stained using antibodies against CD34, Flk-4, OX62, ED1, and α-smooth muscle actin (α-SMA), and then observed under a confocal microscope.

**MATERIALS AND METHODS**

Forty-five neonatal and adult Wistar rats were the subjects of this research (Table 1). All animals were kept in an air-conditioned environment and given water and laboratory chow *ad libitum*. All surgical and experimental procedures were reviewed and approved by the Animal Experiment Committee of Toyama Medical and Pharmaceutical University.
Fig. 2a and b. Confocal micrographs of the diaphragm of a rat intraperitoneally injected with Dil-ac-LDL and transcardially infused with FITC-gelatin. Lymphatic vessels (L, red) can clearly be distinguished from blood vessels (green). a Arteriole, C capillary. Postnatal day 1.

Fig. 3. Confocal micrographs of a rat diaphragm showing Dil-ac-LDL-labeled cells. Postnatal day 1.

a. Numerous free cells labeled with Dil-ac-LDL are seen between lymphatic vessels (L) with tapered ends (arrowheads), suggestive of sprouts. b. A number of free Dil-ac-LDL-labeled cells (arrows) are shown in association with labeled cells incorporated in the lymphatic vessels (L).

Table 1. Animal groups used for the present research.

<table>
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<th>Age</th>
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Antibodies

The primary antibodies used were goat anti-human CD34 and rabbit anti-mouse Flt-4 (1:1000 and 1:250 respectively, Santa Cruz, USA), mouse anti-rat dendritic cells (OX62, 1:100, Serotec, Raleigh, UK), mouse anti-rat macrophages/monocytes (ED1, 1:200, BMA Biomedicals AG, Augst, Switzerland), and mouse anti-α-SMA (1:100, NeoMarkers, Fremont, USA). The secondary antibodies used were biotinylated rabbit anti-goat immunoglobulins (1:400,
DAKO, Glostrup, Denmark), biotinylated goat anti-rabbit immunoglobulins (1:300, DAKO), and biotinylated goat anti-mouse immunoglobulins (1:200, DAKO).

DiI-ac-LDL injection
DiI-ac-LDL (Biomedical Technologies, Stoughton, USA) was diluted with Dulbecco Eagle minimum essential medium (1:100). Under anesthesia with diethyl ether, the rats were intraperitoneally injected with 20 μl/g of the solution. After 4 h survival, the rats were euthanized by inhaling diethyl ether. The diaphragm with the thorax was excised from each rat, and then immersed in 4% paraformaldehyde in 0.01 M phosphate buffered saline (PBS, pH 7.2–7.4) at 4°C for 20 min. The diaphragm was removed from the thorax and washed for 5 min each with five changes of PBS. Some specimens were observed under a confocal microscope (Olympus LSM-GB200, Olympus, Tokyo), while others were processed for immunohistochemistry.

FITC-gelatin infusion
After euthanasia, some rats that had been injected intraperitoneally with DiI-ac-LDL were transcardially infused with FITC-gelatin. FITC-gelatin was prepared according to the method described by HASHIMOTO and KUSAKABE (1996). After the rats had been cooled in a freezer for about 30 min, their diaphragms were excised and fixed in 10% formalin. Each diaphragm was then observed under a confocal microscope.

Immunohistochemistry
Immunohistochemistry was performed by the ABC method. The fixed diaphragm was incubated with primary antibodies against CD34, Flt-4, OX62, and ED1 in PBS containing 3% bovine serum albumen (BSA) at 4°C overnight. After washing with five changes of PBS, it was incubated with the secondary antibody in PBS containing 3% BSA at room temperature for 2 h, washed with five changes of PBS, and incubated with FITC-conjugated streptavidin (1:50, DAKO) for 1 h in a dark moisture chamber. After washing with three changes of PBS, it was examined under a confocal microscope.

For immunohistochemistry for α-SMA, the fixed diaphragm was soaked in PBS containing 0.5% Triton X-100 for 2 h. After washing for 5 min each with three changes of PBS, it was incubated with 10% goat serum (Histofine SAB-PO Kit, Nichirei, Tokyo) for 1 h, washed for 10 min each with three changes of PBS, and incubated with anti-α-SMA antibody in 3% BSA at 4°C for 48 h. The diaphragm was then reacted with the secondary antibody and FITC-conjugated streptavidin in the same way as above. Observations were made under a confocal microscope.
RESULTS

Endothelial cells of morphologically identifiable lymphatic vessels took up intraperitoneally injected Dil-ac-LDL (Figs. 1, 2), while transcardially infused FITC-gelatin filled the blood vessels (Fig. 2). Thus, confocal microscopy of rat diaphragms intraperitoneally injected with Dil-ac-LDL and transcardially infused with FITC-gelatin simultaneously displayed lymphatic vessels in red and blood microvessels in green (Fig. 2). Thicker arterioles were usually accompanied by one or two venules, while thinner ones coursed independently from the venules. Blood capillaries formed dense networks to surround muscular fibers. Lymphatic vessels were generally much thicker than the blood capillaries. Most of the lymphatic vessels ran independently of the blood vessels, except for thicker ones that coursed along the thicker arterioles and venules.

During postnatal week 1, many free cells as well as endothelial cells of pre-existing lymphatic vessels were labeled by Dil-ac-LDL in the diaphragm. The Dil-ac-LDL-labeled free cells were especially abundant around apparently developing lymphatic vessels with tapered ends, and were frequently in line and parallel with the lymphatic vessels (Figs. 3, 4). Many of the Dil-ac-LDL-labeled cells were in contact with the slender and irregular lymphatic vessels that appeared to be sprouts (Fig. 3b). Occasionally, there
were saccular or tubular structures formed of the Dil-ac-LDL-labeled cells (Fig. 4). Since these structures were isolated from the distinct lymphatic vessels, we referred to them as “lymphatic islands”.

Dil-ac-LDL-labeled cells in the diaphragm showed immunoreactivity for CD34 and Fli-4 (Figs. 5, 6a). Endothelial cells of the distinct lymphatic vessels in the diaphragm also showed CD34 and Fli-4 immunoreactivities. However, the majority of Dil-ac-LDL-labeled cells did not express immunoreactivity for either OX62 or ED1, although some of the Dil-ac-LDL-labeled cells expressed ED1 immunoreactivity (Figs. 6b, 7).

By the end of postnatal week 1, the lymphatic network in the diaphragm assumed an organization similar to that in the adult rat: lattice-like lymphatic lacunae with irregular and wide lumina and without valves developed in the subperitoneal region, while a polygonal network of saccular-tubular lymphatic vessels with valves extended into the subpleural region (Fig. 1a, b). However, as the diaphragm enlarged, lymphatic vessels also increased until postnatal week 6. After postnatal week 1, the number of free cells labeled by Dil-ac-LDL in the diaphragm almost disappeared, although some macrophages that had taken up Dil-ac-LDL were observed in organs such as the spleen, liver, kidney, and heart.

Towards the end of postnatal week 2, isolated fusiform cells expressing α-SMA immunoreactivity increased in the diaphragm (Fig. 8a). Some of them appeared to be in contact with lymphatic vessels (Fig. 8b). At postnatal week 2, as the number of these free cells decreased, a coarse meshwork of α-SMA immunoreactive cells with long slender processes began to appear around the lymphatic vessels that took up Dil-ac-LDL (Fig. 8c–e). In adults, smooth muscle cells with α-SMA immunoreactivity formed dense networks around lymphatic vessels; the smooth muscle cells ran rather circularly around valves, while they tended to course longitudinally or obliquely between them (Fig. 8f).

**DISCUSSION**

The present study has demonstrated that lymphatic endothelial cells in the diaphragm take up intraperitoneally injected Dil-ac-LDL. VOYTA et al. (1984) reported that endothelial cells of capillaries and aortae are labeled with Dil-ac-LDL, and it is known that macrophages as well as endothelial cells take up acetylated LDL via the “scavenger cell pathway” of the LDL metabolism (NETLAND et al., 1985). Although there are no available reports of the in vivo labeling of lymphatic endothelial cells, it is obvious that endothelial cells of lymphatic vessels are labeled with Dil-ac-LDL, because as is shown in Figure 1, the vessels in question reveal every morphological fea-
ture of lymphatic vessels, and none of blood vessels. Thus, the present method appears to be most useful to demonstrate lymphatic vessels in the diaphragm. Whether this method can be applicable to other organs is now being investigated in our laboratory.

How does intraperitoneally injected Dil-ac-LDL preferentially proceed to the lymphatic vessels in the diaphragm? In 1863 von Recklinghausen first described how intraperitoneally injected milk entered the diaphragmatic lymphatic vessels. It is now well known that lymphatic vessels in the diaphragm directly open to the peritoneal cavity through lymphatic stomata (Bettendorf, 1978, 1979; Leak and Rahil, 1978; Tsilibary and Wissig, 1983, 1987; Ohtani et al., 1993; Nakatani et al., 1996; Ohtani and Ohtani, 1997). Thus, it may be natural to conceive that Dil-ac-LDL injected into the peritoneal cavity entered the diaphragmatic lymphatics through those stomata to be taken up by endothelial cells of the vessels. In neonatal and young rats, however, the lymphatic stomata are not well developed (Nakatani et al., 1996; Shao et al., 1998). It is thus reasonable to presume that intraperitoneal fluids might enter the interstitial space of the diaphragm.
**Fig. 8.** Confocal micrographs showing α-SMA immunoreactive cells in a rat diaphragm.  

**a.** At postnatal day 1, there are many fusiform α-SMA immunoreactive cells (green).  
**b.** Some α-SMA immunoreactive cells (arrows, yellow) are in contact with lymphatic vessels (L) labeled with DiI-ac-LDL (red). Free cells immunoreactive for α-SMA are also seen (arrowheads).  
**c.** Postnatal day 14. Irregularly shaped α-SMA immunoreactive cells (S, green-yellow) are attached to a lymphatic vessel labeled with DiI-ac-LDL (L, red).  
**d.** Postnatal day 10. A coarse network of α-SMA immunoreactive cells with long, slender processes (S, green-yellow) surrounding a lymphatic vessel labeled with DiI-ac-LDL (L, red).  
**e.** Postnatal week 3. A lymphatic vessel partially labeled with DiI-ac-LDL (red) is surrounded by a coarse network of α-SMA immunoreactive cells (S, yellow-green).  
**f.** Postnatal week 6. A network of α-SMA immunoreactive cells surrounding a thick lymphatic vessel. The α-SMA immunoreactive cells run circularly around presumable valves (arrows), while tending to run obliquely or longitudinally between them. Postnatal week 6.
through gaps among mesothelial cells as well as through incomplete lymphatic stomata.

Hashimoto and Kusakabe (1996) reported a method for the infusion of fluorescent-conjugated gelatin into the blood vascular system for confocal microscopy. In the present study, we infused FITC-gelatin into the blood vascular systems of rats 4 h after they were intraperitoneally administered with DiI-ac-LDL, and simultaneously showed blood and lymphatic vessels in the diaphragm in different colors. This method has greatly facilitated the differential demonstration of both vessels in the diaphragm.

It is noteworthy that there were many DiI-ac-LDL-labeled free cells around lymphatic vessels in the diaphragm during postnatal week 1. Most of these cells showed immunoreactivity for CD34 and Flt-4, but not for OX62 or ED1. CD34 is a 105–129 kDa transmembrane glycoprotein expressed on hematopoietic progenitor cells, vascular endothelial cells, and some fibroblasts. Sauter et al. (1998) reported that CD34 is expressed on lymphatic capillaries in normal human skin. Flt-4, which is suggested to be a receptor for VEGF-C, is initially expressed in angioblasts of the murine head mesenchyme, dorsal aorta, cardiac vein and allantois, and thereafter its expression becomes restricted to lymphatic endothelial cells (Kaipainen et al., 1995; Dumont et al., 1998). A mouse immunoglobulin G1 monoclonal antibody OX62 (or MRC OX62) can be raised against density gradient-enriched rat veiled (dendritic) cells obtained from lymph (Brennan and Puklavc, 1992). ED1 is widely used as a marker for rat macrophages (Damoiseaux et al., 1994). In this context, it is likely that the DiI-ac-LDL-labeled cells are mostly lymphatic endothelial cells and in part blood vascular endothelial cells, but not dendritic cells, although some labeled cells appear to be macrophages.

Thus, the finding that DiI-ac-LDL-labeled free cells exist in abundance around developing lymphatic vessels strongly suggests that migrating lymphatic endothelial cells also participate in lymphangiogenesis in the diaphragm. This is consistent with our previous findings of 5'-Nase positive cells around lymphatic vessels in the diaphragm of late embryonic and neonatal rats (Shao et al., 1998). Endothelial cells of lymphatic vessels contain richer 5'-Nase than those of blood vessels (Werner et al., 1987). Our previous study also showed that lymphatics in the diaphragm primarily develop by sprouting from previously constructed lymphatic vessels (Shao et al., 1998). It is, therefore, reasonable to note that lymphatic vessels in the diaphragm are formed by migrating endothelial cells as well as by sprouting from previously formed vessels.

Interestingly, there were occasional lymphatic islands isolated from morphologically identifiable lymphatic vessels. Additionally, abundant, presumably endothelial, cells were in line and some were in contact with lymphatic islands. This supports our hypothesis that migrating endothelial cells might participate in the construction of new lymphatics. It is presumed that migrating lymphatic endothelial cells form lymphatic islands in the area where lymphatic vessels should be constructed. These islands then elongate by incorporating migrating endothelial cells and become connected with other lymphatic islands and finally with lymphatic vessels that have already constructed a network.

Free cells expressing α-SMA in the diaphragm increased towards the end of the postnatal week 2. Furthermore, at postnatal week 2, α-SMA immunoreactive free cells gathered around lymphatic vessels, and most of them appeared to be in association with lymphatic vessels. At this stage, coarse networks of α-SMA immunoreactive cells with long processes began to appear around thicker lymphatic vessels. These findings suggest that mesenchymal cells differentiated into smooth muscle cells, which in turn came to embrace lymphatic vessels. The smooth muscle cells surrounding lymphatic vessels seem to further proliferate with age to form a dense meshwork. The three-dimensional organization of lymphatic smooth muscle cells in the diaphragm resembles that in other organs (Ohtani, 1992).

REFERENCES


